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Site-directed mutagenesis of histidine 62 in the 'basic patch' region of yeast phosphoglycerate kinase

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Site-directed mutagenesis has been used to produce a mutant form of yeast phosphoglycerate kinase (PGK) in which the 'basic patch' residue His 62 has been replaced by a glutamine residue Using ^{1}H-NMR spectroscopy, it was found that 3-phosphoglycerate (3-PG) binding to the mutant protein induces the same conformational effects as for wild-type PGK, although the affinity was reduced by 2- to 3-fold Kinetic studies show both K_m for 3-PG and V_{max} to be increased by ~2-fold relative to the wild-type enzyme These data are consistent with the suggestion that His 62 assists in the binding of the substrate to the enzyme

Nuclear magnetic resonance, 3-Phosphoglycerate, Phosphoglycerate kinase, Site-directed mutagenesis, Substrate binding

1. INTRODUCTION

The X-ray structure determination of the yeast glycolytic enzyme phosphoglycerate kinase (PGK) indicates that two basic residues are positioned with respect to the triose substrate, 3-phosphoglycerate (3-PG), such that they must almost certainly interact with the non-transferable phosphate during the catalytic reaction [1,2]. More recent ^{1}H-NMR results suggest that 3-PG forms a hydrogen bond, for part of the time at least, with His 62 [3]. Further interactions of the 3-phosphoryl moiety of 3-PG with Arg 168 have also been suggested from the results of site-directed mutagenesis studies [2,3], and with Arg 65 from results of paramagnetic anion binding studies [4]. Binding of 3-PG was observed to cause specific short- and long-range conformational changes which were characterised by various chemical shift changes and broadening effects in the ^{1}H-NMR spectrum [3,5]. A critical role was attributed to the totally conserved Arg 168 residue in propagating the 3-PG-induced conformational changes [3]. His 170, on the other hand, was demonstrated to be of little or no importance in the binding or reaction of 3-PG [3].

In our continuing effort to understand the role in both substrate binding and catalysis of the basic residues close to the active site, a further site-specific mutant form of yeast PGK has been produced. The mutation involves the replacement of conserved amino acid His 62 with a glutamine residue (H62Q). Here we report on the properties of this mutant enzyme as determined kinetically and by ^{1}H-NMR spectroscopy.

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2. MATERIALS AND METHODS

Wild-type PGK and mutant H62Q were prepared as previously described for mutants R168K and R168M, using an over-producing yeast strain (MD40/4C) transformed with the expression vector pYE-PGK [2,6]

Extraction and purification of the over-expressed proteins were carried out essentially as described in [2] and [6] The protein concentration was determined using an absorption coefficient of $A^{1\%}_{1cm} = 4\ 9$ at 278 nm [7].

For kinetic measurements, the protein was further purified by FPLC on a Superose 12 column run in Bistris buffer, pH 6.5, to remove any trace of sulphate in the preparation Samples for NMR experiments, in the concentration range 1–2 mM, were prepared in 100 mM sodium d_3-acetate/D_2O, pH 7 10 ± 0 05 as previously described [3,5]

ATP and 3-PG were obtained from Sigma Chemical Company and NADH was obtained from Boehringer Mannheim GmbH *Bacillus stearothermophilus* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was kindly supplied by Dr A Wonacott (Imperial College, London) All other chemicals used were of AnalaR grade

Kinetic constants, K_m and V_{max}, were determined for the reaction of PGK with the substrate 3-PG using a coupled assay with GAPDH [2] The initial velocities were expressed as ΔA_{340} min^{-1} for NADH at 25°C The assay mixture contained 30 mM triethanolamine/HCl buffered at pH 7 5, 40 mM $(NH_4)_2SO_4$, 0 15 mM NADH, 5 mM ATP and approx 1 μg/ml GAPDH from *B stearothermophilus* The concentration of free Mg^{2+} was maintained constant at 1 mM [8], while that of 3-PG was varied

The effects of sulphate on the activity of the mutant enzyme were examined and compared with the wild-type enzyme The ammonium sulphate concentration was varied from 0–50 mM and the Mg-ATP and 3-PG concentrations were both 0 5 mM The other assay conditions were as above

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One-dimensional ^{1}H-NMR spectra were recorded either on a Bruker AM500 or AM600 spectrometer, at a temperature of 27°C Chemical shift values were determined using acetone as an internal reference at 2 214 ppm Prior to Fourier transformation, the free induction decays were zero-filled from 8192 to 16384 data points, and a resolution-enhancement Gaussian multiplication was applied (GB = 0 10, LB = −10 Hz) The peaks in the ^{1}H-NMR spectra are labelled according to [3] and the resonance assignments used have been previously reported [3,5]

^{1}H-NMR spectral titrations of mutant H62Q with 3-PG were carried out as previously described for wild-type PGK [3] The dissociation constant, K_d, was determined from the change in chemical shifts of resonances assigned to the remaining 'basic patch' histidines, 167 and 170, as a function of the 3-PG H62Q molar ratio

3. RESULTS AND DISCUSSION

3.1. *Structural integrity of mutant H62Q*

Comparison of the aromatic region of the ^{1}H-NMR spectrum of H62Q with that of wild-type PGK (fig.1) reveals few differences. The most marked effect is the absence of resonance 3 in the spectrum of the mutant protein, thus confirming the previous specific assignment of this peak to the C2-H of His 62 [3]. Peak 5a, assigned to His 170 [3], is seen to have shifted downfield (~0.1 ppm). The shortest distance between His 62 and His 170 in the crystal structure of yeast PGK [1] is ~5 Å. The only other significant effects (as observed in a difference spectrum) are small shifts (≤0.01 ppm) of resonances assigned to 'basic patch' histidines 167 (peaks 4 and 15b) and 170 (peak 13a), and of an unassigned component of peak 14a. There is no evidence from the difference spectrum to enable the assignment of a specific peak to the C4-H of His 62.

In the aliphatic region of the spectrum of H62Q (not shown) there are no effects which can be attributed directly to the introduced glutamine residue or to the loss of the substituted histidine, although there are some effects observed in the methyl region. These include effects at peaks 32a (1.26 ppm), 32b (1.21 ppm), 37b (0.28 ppm) and 42 (−0.56 ppm) as numbered in the spectrum of wild-type PGK [3]. Each of these peaks is also affected by 3-PG binding to the wild-type protein and peak 32a gives an NOE connectivity to resonances assigned to His 62 (peak 3) and His 167 (peak 4) [3].

The small number of effects observed on substitution of His 62 with a glutamine residue indicates that the overall conformation of the mutant protein in solution is very similar to that of wild-type PGK. The effets observed are due to local environmental changes in the vicinity of the substitution.

3.2. *Binding of 3-PG*

The effect of 3-PG addition on the spectrum of H62Q is also very similar to that observed for wild-type PGK [3] (fig.2), indicating that the shift in conformational equilibrium induced by triose binding to this mutant and to the wild-type enzyme are similar in both cases. These conformational changes are thought to involve the relative movements of at least three helices [5].

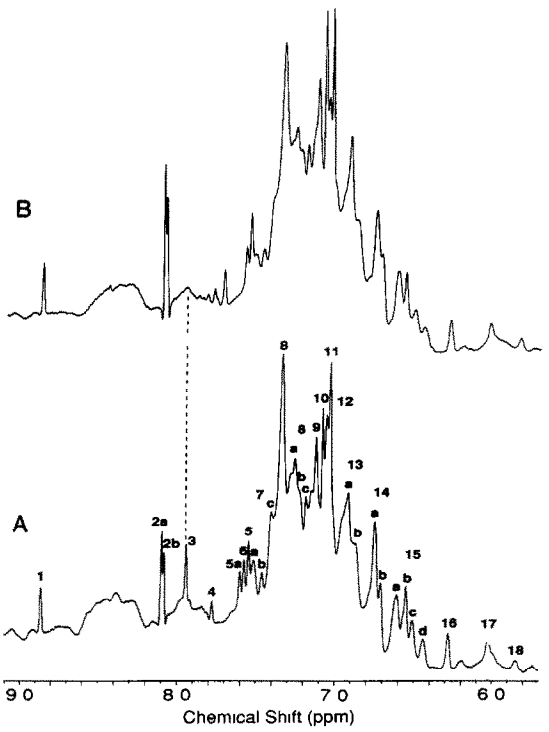

Fig 1 Comparison of the aromatic regions of the 600 MHz ^{1}H-NMR spectra of wild-type (A) and H62Q (B) PGK at pH 7 1

The dissociation constant for 3-PG binding was calculated by fitting the observed changes in chemical shifts of resonances 4 and 15b (fig.3), corresponding to His 167. The shifts of these two resonances saturate with a dissociation constant K_d = 0.029 ± 0.009 mM, which is only a factor of ~3 greater than that obtained from similar titrations of the over-expressed wild-type enzyme (0.011 ± 0.005 mM [3]).

The observed reduction in affinity is too small to suggest the loss of a full hydrogen bond [9]. It is possible, however, that the side-chain of Gln 62 will have a similar interaction with the bound substrate as that proposed [3] for the histidine it has replaced. It is noted that while His 62 is a totally conserved residue in the 22 primary sequences now available, the only observed (active) natural mutation of 'basic patch' His 167 is to a glutamine residue (in PGK from *Penicillium chrysogenum* [10]).

3.3. *Catalytic properties*

The kinetic data from mutant H62Q are compared with those from the wild-type enzyme in table 1. Both the K_m for 3-PG and the V_{max} have increased relative to the wild-type enzyme. The small increase in K_m is consistent with the observed small increase in K_d for 3-PG. The NMR observations of 3-PG binding (above) suggest that the substrate will be correctly positioned for phosphoryl transfer from Mg·ATP. To explain the in-

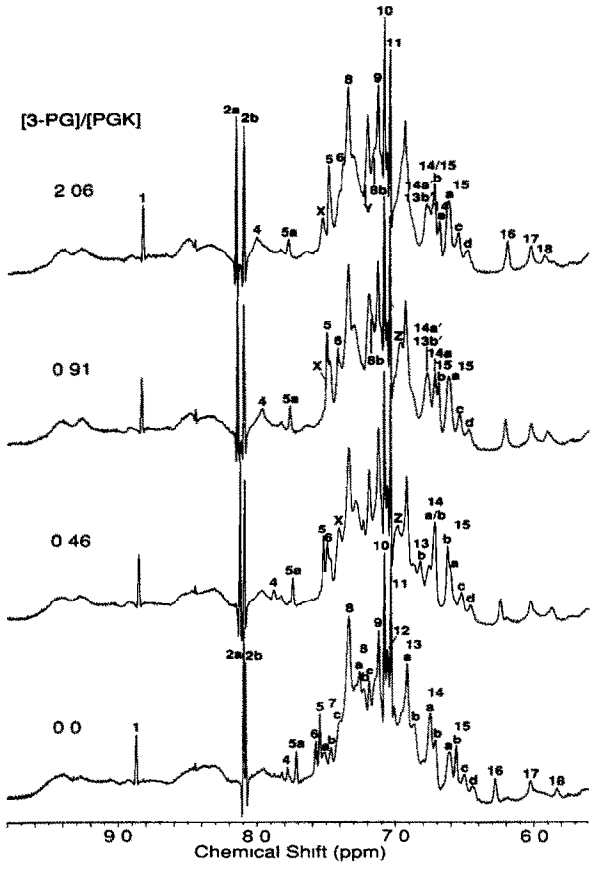

Fig.2. Perturbations of the aromatic region of the 500 MHz ^{1}H-NMR spectrum of mutant H62Q PGK by 3-PG, at the ratios indicated and pH 7 1

creased turnover of this enzyme relative to wild-type PGK, it is necessary to assume that the binding of 1,3-bisphosphoglycerate (1,3-P_2G) has also been reduced by a factor of ~2. Since dissociation of this substrate is the rate determining step in the catalytic reaction [11], increasing its dissociation constant by a factor of ~2 would increase the overall rate by a similar factor, assuming that the rate of phosphoryl transfer remains unaltered.

Anions have been shown to act as activators of PGK at low concentrations, but they inhibit at higher concentrations [8,12]. Experiments carried out with mutant H62Q indicate that the effect of sulphate is the same for both the mutant and wild-type enzymes.

Table 1

Michaelis-Menten kinetic parameters for mutant H62Q and wild-type yeast PGK with 3-PG

Enzyme	V_{max} (EU mg^{-1})	K_m (mM)
Wild-type	460	0 78
H62Q	1062	1 89

The enzyme activity (EU) is expressed as 1 μmol NADH converted/min at 25°C

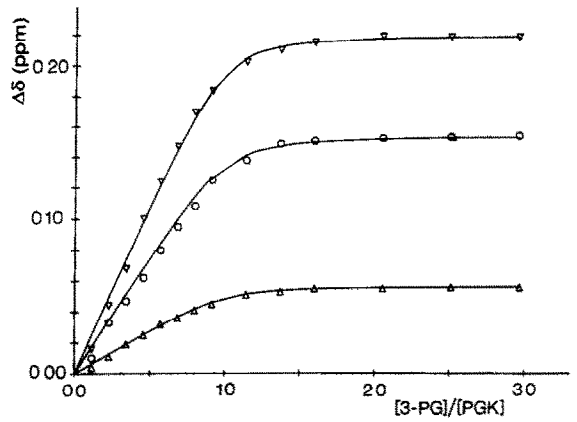

Fig 3 The change in the chemical shifts of peaks 4 (▽ ; His 167), 5a (△ , His 170) and 15b (○, His 167) plotted as a function of the 3-PG H62Q concentration ratio The continuous lines represent theoretical binding curves corresponding to a dissociation constant K_d = 0 029 mM

4. CONCLUSIONS

Histidine 62 is a conserved residue throughout all known PGK sequences (see [3] and references therein and [10,13]). The pK_a of this group has not been determined but it is known from NMR studies to lie below 3 [3]. This is not within the range of histidine pK_a values found for those active site histidines which act as proton donors/acceptors and therefore its role in catalysis cannot be to provide an acid-base centre. An alternative possible function is that it acts as a hydrogen-bonding centre assisting in binding of the substrate, 3-PG. Support for this comes from the crystal structure determination [1,2], from the observation of a large downfield shift (>0.7 ppm) of resonance 3 in the ^{1}H-NMR spectrum on binding 3-PG (indicating partial protonation of the histidine) [3] and from its position relative to the observed general anion binding site [4]. In the mutant H62Q, we find that the binding of the substrate is somewhat reduced and the overall catalytic rate enhanced, which is in keeping with an altered (reduced) hydrogen-bonding strength from residue 62. It is also consistent with the X-ray observation [1] that in the 'open' form of the structure, His 62 is correctly orientated to form a hydrogen bond with the phosphoryl moiety of 3-PG, although it is too far away (~5 Å) to do so.

The results of sulphate activation experiments suggest that His 62 is of less significance in general anion binding, consistent with the small shifts (~0.03 ppm) observed for resonance 3 in ^{1}H-NMR studies of sulphate (and phosphate) binding [4].

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